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Characteristics and Polyadenylate Content of the Actin Messenger RNA of Mouse Sarcoma-180 Ascites Cells†

Thomas E. Geoghegan,* Gail E. Sonenshein,‡ and George Brawerman

ABSTRACT: Actin is a major protein component of mouse sarcoma-180 ascites cells. It is produced in large amounts in a wheat germ cell-free system supplemented with total polysomal RNA from these cells. Adsorption of the poly(A)$^+$ RNA onto oligo(dT)-cellulose leads to the retention of the template activity for most polypeptides but leaves a substantial portion of the actin mRNA in the unadsorbed fraction. The actin mRNA that binds to oligo(dT)-cellulose contains a large proportion of chains unable to bind to Millipore filters. The other major poly(A)$^+$ mRNAs bind nearly as well to Millipore filters as to oligo(dT)-cellulose. This implies that the distribution of poly(A) sizes in the actin mRNA is atypical, with a large proportion of the chains having relatively short poly(A) segments and with many chains containing either very short segments or no poly(A) at all. The translation of actin mRNA is preferentially inhibited in the presence of excess poly(A)$^+$ RNA. Both the poly(A)-containing and poly(A)-deficient forms of actin mRNA exhibit this sensitivity to inhibition of translation. Inhibitors of polypeptide chain initiation such as poly(A) or poly(U) did not inhibit preferentially actin mRNA translation. The poly(A)$^-$ actin mRNA appears to be functional in the cell, since it is found associated with polysomes in cytoplasmic extracts. A 26-fold enrichment in the poly(A)-deficient actin mRNA was achieved by first isolating a 50S ribonucleoprotein particle from (ethylenedinitrilo)tetraacetic acid treated polysomes and subjecting the deproteinized material to oligo(dT)-cellulose fractionation, followed by zone centrifugation.

The physiological significance of the poly(A)[1] sequence at the 3′ end of eukaryotic mRNA chains remains poorly understood. This segment is present in most mRNAs characterized so far but is absent from the RNA species that code for histones (Adesnick & Darnell, 1972; Greenberg & Perry, 1972). Comparisons between the histone mRNAs and poly(A)-containing species have failed to reveal significant functional differences that might be attributed to this sequence. The histone mRNAs do not appear to be particularly unstable (Perry & Kelley, 1973) nor do they seem to have any unusual requirements for translation. Removal of the poly(A) from normally polyadenylylated species, however, leads to a marked loss of stability when the depleted species are injected into frog oocytes (Huez et al., 1974, 1975). This feature points to a role for the poly(A) in the control of mRNA stability, but it fails to account for the apparent stability of the histone mRNAs. The status of the latter species is further complicated by the finding that they can also exist as polyadenylylated components in sea urchin eggs (Levenson & Marcu, 1976; Ruderman & Pardue, 1976). The protamine mRNA of developing trout testes has also been shown to occur in both forms (Iatrou & Dixon, 1977).

The study of additional mRNA species that exist without poly(A) could conceivably reveal significant functional features specifically related to this sequence. It is suspected that a substantial portion of the mRNA of mammalian cells lacks a poly(A) segment (Milcarek et al., 1974). We observed that the polysomal RNA of certain mammalian tumor cells depleted of poly(A)-containing species can promote the synthesis of a major polypeptide of 45 000 molecular weight in wheat germ extracts (Sonenshein et al., 1976). This polypeptide has been identified as actin by several investigators (Hunter & Garrels, 1977; Kaufmann et al., 1977). In the present report we confirm the identification of this polypeptide as actin and provide evidence that it exists as polyadenylylated and non-polyadenylylated forms, both apparently functional in the intact cells. The two types of actin mRNA chains show the same characteristics of translation in vitro. It is possible that

† From the Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111. *Received April 25, 1978.* This work was supported by research grants from the United States Public Health Service (GM 17873) and the National Science Foundation (BMS 72-02265).

‡ Current address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

[1] Abbreviations used are: poly(A), polyadenylic acid; poly(U), polyuridylic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; ATP and GTP, adenosine and guanosine triphosphates; oligo(dT), oligodeoxythymidylate; DNase, deoxyribonuclease I (EC 3.1.4.5); EDTA, (ethylenedinitrilo)tetraacetic acid.

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the poly(A)-deficient actin mRNA is derived from the poly(A)-containing species and that it remains functional in the cells after loss of this sequence. This would be in contrast to the behavior of the other poly(A)-containing mRNAs that do not normally accumulate in the cells after loss of the poly(A) segment.

Experimental Procedures

Cell Incubations and Preparation of Polysomes and RNA. Mouse sarcoma-180 cells were maintained by weekly transfer into the peritoneal cavity of male albino mice. Cells were harvested from animals 6–8 days after inoculation, incubated in full medium for 1 h, and lysed with Triton X-100 at low ionic strength as described previously (Mendecki et al., 1972). Polysomes were obtained from the cytoplasmic fraction by magnesium precipitation (Mendecki et al., 1972), resuspended in 50 mM KCl, 1 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.6), and stored in liquid nitrogen or at −85 °C. RNA was prepared by the phenol-extraction procedure at alkaline pH (Brawerman et al., 1972) modified as follows. After an initial extraction with phenol, and a reextraction of the phenol phase with 0.1 M Tris-HCl (pH 9.0), the combined aqueous phase was reextracted with a mixture containing phenol, chloroform, and isoamyl alcohol in ratios of 1:1:0.1. This procedure was repeated until the interface was free of protein. RNA was precipitated overnight at 4 °C with the addition of NaCl to 0.1 M and 2.5 volumes of ethanol, collected by centrifugation, and washed with 66% ethanol and 0.1 M NaCl. Excess ethanol was removed by washing the RNA pellet with ether before dissolving in distilled water. Residual ether was removed with a stream of air, and RNA solutions were stored at −20 °C.

Oligo(dT)-cellulose Fractionation. RNA was separated into poly(A)$^+$ and poly(A)$^-$ fractions as follows. The polysomal RNA was mixed at room temperature with oligo(dT)-cellulose (Collaborative Research, type T-3) in the presence of 500 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6), and 0.1% sodium dodecyl sulfate. After 30 min, the mixture was centrifuged and washed two times with the same buffer. The combined supernatant represents poly(A)$^-$ RNA. The adsorbed poly(A)$^+$ material was eluted with successive washes with water. The two RNA fractions were precipitated with ethanol–salt and processed as described above.

Purification of Poly(A)$^-$ Actin mRNA. Polysomes (35 A_{260} units/mL) in 50 mM KCl, 1 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.6) were dissociated by treatment with (ethylenedinitrilo)tetraacetate (40 mM) followed by zone centrifugation through a 35-mL, 10–30% sucrose gradient containing 100 mM NaCl, 1 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.6) in an SW-27 Spinco rotor at 21 000 rpm for 16 h at 4 °C. Under these conditions, the actin mRNA cosediments with the large ribosomal subunit (Sonenshein et al., 1976). Gradient fractions in this region were combined and supplemented with 0.5% sodium dodecyl sulfate, 500 mM NaCl, and 5 mM $MgCl_2$, followed by adsorption on oligo(dT)-cellulose to remove poly(A)-containing material. The nonbound fraction was subjected to alkaline phenol extraction and precipitated with ethanol. The precipitated RNA was redissolved in distilled water at a concentration of 70 A_{260} units/mL and subjected to zone centrifugation through a 35-mL 5–30% sucrose gradient containing 10 mM NaCl, 20 mM Tris-HCl (pH 7.6), and 0.1% sodium dodecyl sulfate, in an SW-27 Spinco rotor at 23 500 for 17 h at 15 °C. This step allows the separation of the actin mRNA, which sediments at 18 S (Sonenshein et al., 1976) from the 28 S ribosomal RNA found in the large ribosomal subunit. Material sedimenting at 18 S was precipitated with ethanol, redissolved in water, and constituted the "purified" poly(A)$^-$ actin mRNA.

Purification of Actin. Cytoplasmic actin was purified from the ascites cells by the method of Yang and Perdue (1972). This involves polymerization of material from a cellular extract in the presence of high salt (100 mM KCl) and ATP at room temperature and depolymerization at 4 °C in low salt medium containing ATP. Actin was also purified by adsorption on DNase covalently linked to Sepharose (Lazarides & Lindberg, 1974). Samples were added to DNase–Sepharose in a mixture containing 500 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.6). After incubation for 30 min at 4 °C, the material was washed twice with the same buffer and twice with a mixture containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.6), and 0.5 M urea. The adsorbed material was eluted by washing with 50 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate and 2 M urea.

In Vitro Protein Synthesis. The reaction mixtures (25 μL) contained 5 μL of wheat germ extract (Roberts & Paterson, 1973), 24 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.2), 0.8 mM spermidine, 1 mM magnesium acetate, 2 mM dithiothreitol, 1.2 mM 2-mercaptoethanol, 1 mM ATP, 0.2 mM GTP, 0.8 mM creatine phosphate, 116 mM potassium acetate, 24 mM KCl, 50 μg/mL creatine phosphokinase, 30 μM each of the common amino acids except methionine, 10 μCi of [^{35}S]methionine (500 Ci/mmol, New England Nuclear), and the indicated amounts of exogenous RNA. After incubation at 22 °C for 2 h, pancreatic ribonuclease A was added to a final concentration of 200 μg/mL, and the reaction mixtures were digested at 37 °C for 30 min. Samples were removed for measurement of hot trichloroacetic acid insoluble radioactivity.

Polyacrylamide Gel Electrophoresis. Proteins were either precipitated with trichloroacetic acid prior to electrophoresis as previously described (Sonenshein et al., 1976) or the reaction mixtures were applied directly to gels after adjusting to 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 35 mM 2-mercaptoethanol, and 10% glycerol. The samples were subjected to electrophoresis on 13% polyacrylamide slab gels (0.8-mm thick) containing sodium dodecyl sulfate, with a 5% polyacrylamide stacking region (Studier 1973). In some experiments, 5.5 and 2.7 M urea was included in the running and stacking regions, respectively (Storti & Rich, 1976). Electrophoresis was for 16 h at 55 V. The gels were stained with Coomassie blue, destained, and dried onto paper in vacuo. Autoradiograms were produced by exposure to Kodak no-screen X-ray film. Quantitative data were obtained by scanning autoradiograms at 540 nm with a Beckman Model DU spectrophotometer adapted with a Gilford gel scanner, followed by manual integration of gel scan peaks.

Results

Identification of Actin in Cell Extracts and Cell-Free Incubation Mixtures. The cytoplasm of the sarcoma cells contains a major polypeptide with a mobility in sodium dodecyl sulfate–polyacrylamide gels corresponding to a molecular weight of 45 000 (Figure 1). This value was determined by comparison with the mobilities of known proteins. Two criteria were used for the identification of this polypeptide as actin. A cell extract subjected to the actin-polymerization procedure followed by depolymerization (Yang and Perdue, 1972) yielded a single polypeptide with the same mobility (Figure 1). This compound was also obtained by specific adsorption to DNase coupled to Sepharose, a characteristic of actin (Lazarides and Lindberg, 1974). A radioactive polypeptide with the same mobility, derived from labeled cell extracts, was

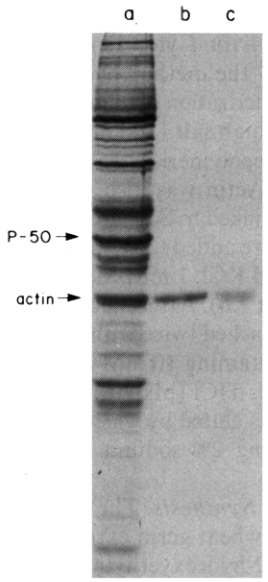

FIGURE 1: Slab-gel electrophoresis of sarcoma-180 proteins. Cytoplasmic proteins, obtained by Triton X-100 lysis of the cells, were subjected to gel electrophoresis in the prsence of sodium dodecyl sulfate and urea, and stained with Coomassie blue: lane a, total cytoplasmic proteins; lane b, proteins bound to DNase–Sepharose and eluted, as described under Experimental Procedures; lane c, proteins subjected to a polymerization–depolymerization cycle in the presence of salt and ATP.

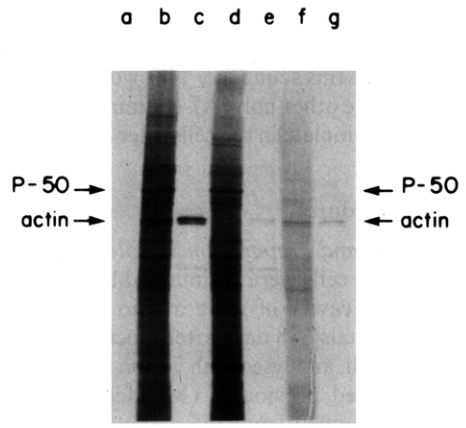

FIGURE 2: Autoradiogram of ^{35}S-labeled polypeptides produced in the wheat germ cell-free system in the presence of ascites RNA fractions. Total cell-free products (lanes a, b, d, and f) and products bound and eluted from DNase–Sepharose (lanes c, e, and g) were subjected to gel electrophoresis in the presence of sodium dodecyl sulfate and urea: Lane a, endogenous cell-free products; lanes b and c, products from total polysomal RNA; lanes d and e, products from poly(A)$^+$ RNA; lanes f and g, products from poly(A)$^-$ RNA.

TABLE I: Effect of Repeated Adsorptions of Polysomal RNA to Oligo(dT)-cellulose on Activity for Actin Synthesis.[a]

	binding		
	1st	2nd	3rd
actin	2330	2000	1975
P-50	2130	2630	2890
actin/P-50	1.1	0.75	0.70

[a] Sarcoma-180 polysomal RNA was bound to oligo(dT)-cellulose as described under Experimental Procedures. Adsorbed material was eluted with water and subjected to two additional cycles of binding and elution. Aliquots were removed prior to each binding, and a 0.4-μg sample of RNA was used to direct translation in vitro. Products were analyzed on sodium dodecyl sulfate slab gels as described under Experimental Procedures. Values represent amount of polypeptide synthesized in arbitrary units per microgram of RNA, obtained by scanning of autoradiograms.

recovered after binding to DNase–Sepharose. The actin showed a tendency to separate into two bands in gels supplemented with urea (see Figures 1 and 2).

The 45 000-dalton polypeptide is a major translation product of sarcoma cell polysomal RNA in the wheat germ cell-free system (Sonenshein et al., 1976). The cell-free product could also be recovered after binding to DNase–Sepharose (Figure 2). Both the poly(A)$^+$ and the poly(A)$^-$ RNA fractions, separated by oligo(dT)-cellulose fractionation, were active in actin synthesis. The additional band with higher mobility in the material bound to DNase–Sepharose (Figure 2) was not always observed.

Poly(A) Content of Actin Messenger RNA. It was shown previously that oligo(dT)-cellulose fractionation of the sarcoma polysomal RNA left mRNA for the 45 000-dalton polypeptide, shown here to represent actin, in the unadsorbed fraction (Sonenshein et al., 1976). Repeated readsorptions of this fraction failed to remove the activity for actin synthesis. Thus, the active material in this fraction seemed devoid of poly(A). Some activity for actin synthesis, however, was present in the oligo(dT)-bound fraction (Sonenshein et al., 1976). In order to determine whether this latter material did, in fact, represent a poly(A)-containing mRNA species, the oligo(dT)-bound fraction was subjected to two additional cycles of binding to this adsorbent. The residual activity for actin synthesis was compared to that for another major polypeptide with a mobility corresponding to a molecular weight of 50 000, designated as P-50 (see Figures 1 and 2). The P-50 mRNA is exclusively a poly(A)-containing species, since no activity for P-50 synthesis is detected in the unadsorbed RNA after oligo(dT)-cellulose fractionation (Figure 2). As can be seen in Table I, the specific activity for P-50 synthesis increased somewhat upon the rebinding of the oligo(dT)-bound material, probably as a result of the removal of residual ribosomal RNA. The specific activity for actin synthesis showed a small decrease upon rebinding. Little further changes in the activities for actin and P-50 synthesis occurred after an additional cycle of binding and elution (Table I). Thus, the residual actin mRNA bound to oligo(dT)-cellulose does represent a genuine poly(A)-containing species.

Poly(A)-containing RNA is known to bind to Millipore filters (Lee et al., 1971), but it is believed that only poly(A) segments of relatively large size can bind to this adsorbent (Gorsky et al., 1974). Thus, a comparison of mRNA yields after Millipore filter and oligo(dT)-cellulose binding should provide a measure of relative distributions of poly(A) sizes. We observed that the material bound to Millipore filters, although highly active as template for protein synthesis, had relatively little activity for actin synthesis. Table II shows that the P-50 mRNA binds nearly as well to Millipore filters as to oligo(dT)-cellulose. The binding of actin mRNA, on the other hand, is considerably less effective on Millipore filters. The unadsorbed material after Millipore filtration contains twice as much activity for actin synthesis as does the flow-through after oligo(dT)-cellulose fractionation. These results indicate that a large proportion of the poly(A)-containing actin mRNA chains have relatively short poly(A) segments. The mRNA activities in the unfractionated RNA and in the unadsorbed fraction are directly comparable, since they are expressed as activity per microgram of total polysomal RNA. It is possible to make some rough estimates of poly(A) size distribution by comparing these activities (Table II). According to these es-

TABLE II: Binding of mRNA for Actin to a Millipore Filter and to Oligo(dT)-cellulose.[a]

RNA fraction	polypept synth	adsorbent: none	adsorbent: Millipore filter	adsorbent: oligo(dT)-cellulose
bound	actin		900	2100
	P-50		675	600
	actin/P-50		1.3	3.5
not bound	actin	150	87	45
	P-50	40	10	
	actin/P-50	3.7	8.7	

[a] Sarcoma-180 polysomal RNA was adsorbed to oligo(dT)-cellulose as described under Experimental Procedures. An identical RNA sample was passed twice through a Millipore filter at room temperature in the presence of 500 mM KCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.6). After washing with same buffer, the RNA bound to the Millipore filter was eluted at 4 °C with 50 mM Tris-HCl (pH 9.0) and 0.5% sodium dodecyl sulfate. Amounts of total RNA and of unadsorbed RNA used to direct translation in vitro were 4 μg, and amounts of adsorbed RNA were 0.4 μg. Translation products were subjected to the sodium dodecyl sulfate–urea gel electrophoresis procedure and processed as described in Table I. Values are expressed as amounts of polypeptide synthesized (in arbitrary units) per microgram of added RNA.

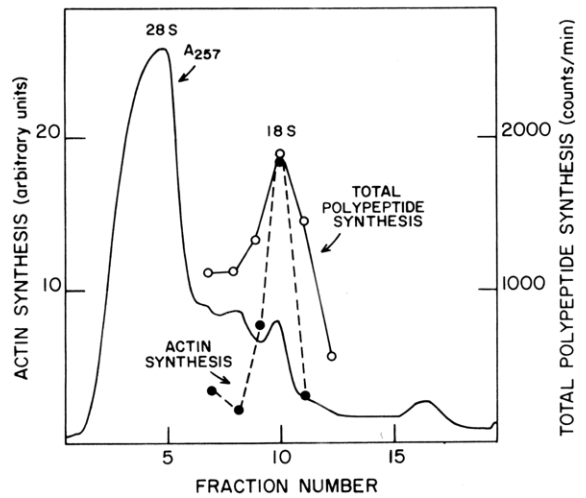

FIGURE 3: Zone sedimentation of actin mRNA. Poly(A)$^-$ RNA (30 A_{260} units), obtained from the large ribosomal subunit region of a sucrose gradient, was centrifuged through a 35-mL 5–30% sucrose gradient in an SW-27 Spinco rotor as described under Experimental Procedures. Tube contents were monitored for absorbance at 257 nm (solid line). Collected fractions were precipitated with ethanol, and samples were used to direct translation in vitro. Total counts per minute, which represents radioactivity insoluble in hot trichloroacetic acid, was determined on aliquots of the incubation mixtures. Values for actin synthesis were determined as described under Experimental Procedures.

timates, about 75% of the P-50 mRNA chains would seem to contain a poly(A) segment large enough to bind to Millipore filters, and the remainder would have a segment of sufficient length to bind to oligo(dT)-cellulose. In the case of actin mRNA, about 40% of the chains would have the large-size poly(A) capable of binding to Millipore filters, 30% of the chains would have a smaller poly(A) segment still capable of binding to oligo(dT)-cellulose, and the remaining 30% would have either a segment too small to bind to oligo(dT)-cellulose or no poly(A) at all. In the present report, the RNA that fails to bind to oligo(dT)-cellulose is designated as poly(A)-deficient or poly(A)$^-$ RNA, and the bound material as poly(A)$^+$ RNA.

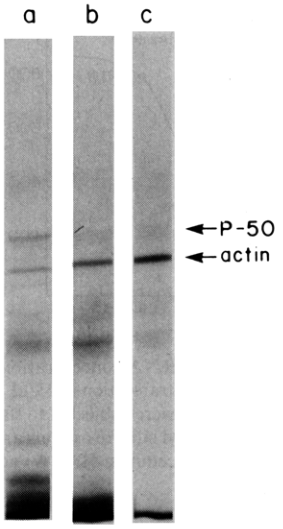

FIGURE 4: Autoradiogram of ^{35}S-labeled polypeptides produced in the wheat germ cell-free system in the presence of various RNA fractions. Cell-free products were subjected to gel electrophoresis in the presence of sodium dodecyl sulfate: (a) 24 μg of total polysomal RNA; (b) 17 μg of poly(A)-deficient RNA; (c) 2 μg of purified actin mRNA. All incubations were in 50-μL volumes.

TABLE III: Partial Purification of Poly(A)-Deficient Actin mRNA.[a]

RNA fractions	actin synthesis (units/μg of RNA)	enrichment
S-180 total polysomal	22	1
poly(A)-deficient, polysomal	36	1.6
poly(A)-deficient, 18 S	81	3.7
purified fraction	580	26

[a] RNA samples were used to direct translation in vitro. Translation products were analyzed as described under Experimental Procedures. Values represent the average from experiments with several RNA concentrations. In each case the amount of actin synthesis showed a linear response to RNA concentration.

Purification of the Poly(A)-Deficient Actin mRNA. Our previous studies had shown that dissociation of polysomes by EDTA treatment releases the actin mRNA as a particle that cosediments with the 50S ribosomal subunit (Sonenshein et al., 1976). The actin mRNA in deproteinized polysomal RNA sedimented as an 18S component. Thus the 50S nucleoprotein complex seemed like a good starting material for the preparation of poly(A)$^-$ actin mRNA free of ribosomal RNA. Figure 3 shows the sedimentation profile of the RNA prepared from the 50S fraction by deproteinization followed by removal of the poly(A)$^+$ species by adsorption to oligo(dT). There is very little 18S ribosomal RNA in this preparation, and the activity for actin synthesis sediments as a homogeneous 18S component. The activity for total polypeptide synthesis shows a much broader sedimentation profile. The 18S fraction, used as "purified" actin mRNA in these studies, showed a 26-fold enrichment in specific activity for actin synthesis (Table III). The level of contaminating mRNA species is also substantially reduced in this fraction, as can be seen in Figure 4 where the distribution of cell-free translation products from different RNA fractions is compared. The increase in specific activity after removal of the poly(A)$^+$ material from the total polysomal RNA shown in Table III was not always observed.

Selective Inhibition of Actin Messenger RNA Translation.

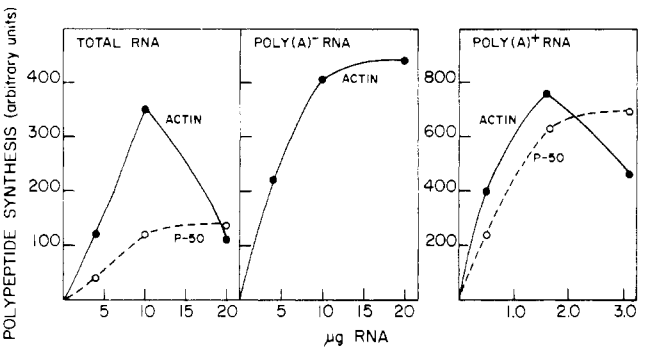

FIGURE 5: Effect of increasing RNA concentrations on actin synthesis. RNA samples were used to direct translation in 25-μL wheat germ reaction mixtures. Products of translation were subjected to slab gel electrophoresis in the presence of sodium dodecyl sulfate and urea, and analyzed as described under Experimental Procedures: (●—●) actin synthesis; (○ - - - ○) P-50 synthesis.

TABLE IV: Inhibition of Actin mRNA Translation in the Presence of Increasing Concentrations of poly(A)-Containing RNA.[a]

	poly(A)-deficient actin mRNA					
	none			3 μg		
poly(A)⁺ RNA	0.5 μg	1.5 μg	3.0 μg	none	0.5 μg	3.0 μg
actin	156	322	192	402	572	86
P-50	100	272	290		175	217
total cpm	4000	11000	7200	1700	6400	4200

[a] Poly(A)-containing RNA was used to direct translation either alone or in the presence of the purified poly(A)-deficient actin mRNA fraction. Translation products were analyzed as described under Experimental Procedures. Values for polypeptide synthesis are expressed in arbitrary units. Total counts per minute represent hot trichloroacetic acid insoluble radioactivity determined on a separate aliquot of incubation mixture. Endogenous incorporation was 340 cpm.

TABLE V: Inhibition of Actin mRNA Translation by Poly(A) and Poly(U).[a]

		% inhibition by homopolymer		
	polypept synth	poly(A) 5 μg	poly(A) 15 μg	poly(U) 10 μg
poly(A)-containing RNA				
actin	1063	64	83	60
P-50	564	60	79	58
total cpm	5915	68	81	58
purif poly(A)-defic actin mRNA				
actin	520	50	78	49
total cpm	1940	57	76	48

[a] Poly(A)-containing RNA (0.8 μg) and partially purified poly(A)-deficient actin mRNA (2 μg) were used to direct polypeptide synthesis in 25 μL of incubation mixtures in the presence or absence of synthetic poly(A) or poly(U). Products were analyzed as described under Experimental Procedures. Values for polypeptide synthesis are expressed in arbitrary units. Total counts per min represent hot trichloroacetic acid insoluble radioactivity determined on a separate aliquot of incubation mixture. Endogenous incorporation was 313 cpm.

The use of saturating amounts of polysomal RNA in the wheat germ cell-free system causes a marked inhibition of actin synthesis (Figure 5; see also Sonenshein et al., 1976). P-50 synthesis is not affected in this fashion. Total polypeptide synthesis is also inhibited but not as extensively as actin synthesis (data not shown). After oligo(dT)-cellulose fractionation, the inhibitory activity remains with the poly(A)⁺ RNA fraction (Figure 5, Table IV). No such inhibition of actin synthesis is observed with the poly(A)⁻ RNA fraction. In order to determine whether the actin mRNA in the latter fraction is susceptible to the inhibitory effect, its translation was examined in the presence of the poly(A)⁺ RNA fraction. When a low level of poly(A)⁺ RNA (0.5 μg) was added to the partially purified poly(A)⁻ actin RNA, the amount of actin produced was roughly the sum of the amounts synthesized from each fraction alone (Table IV). Thus, no inhibition of actin synthesis was apparent in this case. When a higher, saturating, level of poly(A)⁺ RNA was used in conjunction with the poly(A)⁻ actin RNA, an overall inhibition of polypeptide synthesis was observed. Actin synthesis was reduced drastically, to levels well below those expected from the translation of either RNA fraction alone. This result indicates that the translation of the poly(A)⁻ actin mRNA is sensitive to the inhibitory effect of the poly(A)⁺ RNA fraction. It appears, therefore, that the high susceptibility to inhibition of translation is an intrinsic property of the actin mRNA chain, regardless of the presence or absence of a poly(A) segment. The inhibitory agent is not a specific component of actin-producing cells, since the poly(A)⁺ RNA of rat liver can also inhibit actin synthesis when added to the purified actin mRNA (data not shown).

The above results suggested to us that the actin mRNA is unable to compete with other poly(A)⁺ mRNA species when these are present in excess. Thus, the actin mRNA would seem to represent a species relatively ineffective in the initiation process. If this were the case, its translation should be particularly sensitive to inhibitors of initiation, such as synthetic poly(A) or poly(U) (Lodish & Nathan, 1972). Inhibition by poly(A) has been used successfully to evaluate the relative efficiency of translation of certain mRNAs, such as the immunoglobulin light-chain RNA of mouse myeloma cells and the rat liver albumin RNA (Sonenshein & Brawerman, 1976, 1977). However, when inhibitory levels of poly(A) or poly(U) were used in the present translation system, they failed to produce any preferential inhibition of actin synthesis (Table V). Actin synthesis was inhibited to the same extent as P-50 synthesis and as total polypeptide synthesis. The two forms of actin mRNA showed the same response. Thus, the actin mRNA does not appear to be a relatively inefficient species by the criterion of susceptibility to inhibition by the synthetic homopolymers.

Distribution of Actin Messenger RNA in Polysomes and in Smaller Particles. In order to determine whether both the poly(A)⁺ and poly(A)⁻ actin mRNA components are being translated in intact cells, their distribution in polysomes was examined. A cytoplasmic extract was subjected to zone centrifugation, and RNA was prepared from heavy polysomes, light polysomes, particles sedimenting around 80S, and lighter particles (see Figure 6). Because of overloading, the polysomes were not well resolved in this experiment. Parallel centrifugation with less material gave a better resolution of polysomes. From the better resolved profiles, it can be estimated that the light polysome fraction used in these studies ranged from dimers to hexamers.

The products of translation of total RNA, poly(A)⁺ RNA, and poly(A)⁻ RNA from each fraction were compared (Figure 7). It can be seen that translatable poly(A)⁻ actin mRNA was present primarily in the two polysome fractions, with the greatest concentration in the light polysomes. The poly(A)⁺ actin mRNA seemed to be concentrated almost exclusively in the heavy polysomes. Quantitative data on mRNA distribution

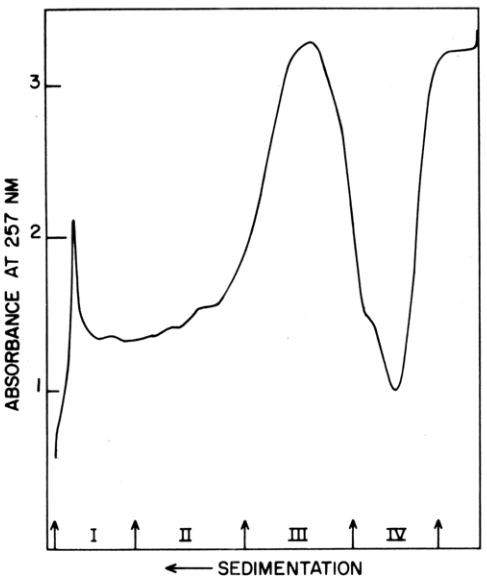

FIGURE 6: Zone sedimentation of cell lysate. Sarcoma cells, lysed with Triton X-100, were subjected to low-speed centrifugation to remove nuclei. The supernatant fraction (3.0-mL samples) was centrifuged in an SW-27 Spinco rotor at 27 000 rpm for 3.5 h through 35 mL of 10–40% sucrose gradients in 100 mM NaCl, 1 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.6), with a 60% sucrose cushion. Gradient fractions were combined as indicated by arrows. Fractions were precipitated with ethanol, deproteinized as described under Experimental Procedures, and used for the experiment shown in Figure 7. The pooled fractions are designated I, II, III, and IV. Yields of RNA were 400 μg for fraction I, 710 μg for fraction II, 3 mg for fraction III, and 470 μg for fraction IV.

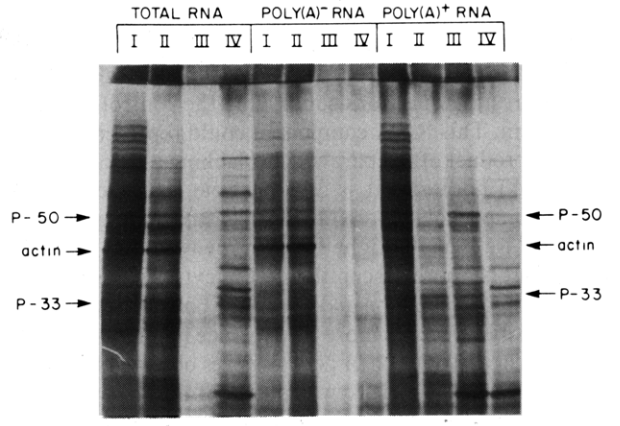

FIGURE 7: Autoradiogram of ^{35}S-labeled polypeptides produced in response to RNA from pooled polysome gradient fractions prepared as described in Figure 6. Samples of pooled fractions were adsorbed to oligo(dT)-cellulose as described under Experimental Procedures. Amounts of RNA used for the cell-free incubations were 4 μg for the total and poly(A)$^-$ fractions and 0.4 μg for the poly(A)$^+$ fractions.

TABLE VI: Distribution of Actin mRNA in Polysome and Small Particle Fractions.[a]

fraction	total polysomal RNA P-50	actin	P-33	poly(A)-defic RNA: actin
I	1450	3350	600	1880
II	1100	4900	570	5500
III	4000	1280	600	1360
IV	1230	180	580	65

[a] Autoradiogram shown in Figure 6 was scanned, and amounts of actin, P-50, and P-33 were determined as described under Experimental Procedures. In order to obtain the distribution of each mRNA activity in the four fractions, the values (in arbitrary units) were multiplied by appropriate factors, taking into account the total amount of RNA in each fraction and the amount added to the wheat germ cell-free system.

in total and poly(A)$^-$ RNA are provided in Table VI. Data on the poly(A)$^+$ RNA were not included because of uncertainity in the quantitative recovery of RNA from the oligo(dT)-cellulose bound fraction. From these data it appears that the light polysomes and the 80S fraction contained only the poly(A)$^-$ form of actin mRNA. The actin mRNA in the light polysome fraction apparently represents RNA associated with polysomes and not free messenger ribonucleoprotein particles. It was shown previously that EDTA treatment of polysomes released the actin mRNA as a 50S nucleoprotein particle (Sonenshein et al., 1976).

The distribution of P-50 mRNA in the various fractions was quite different, with nearly equal amounts in the two polysome fractions and the majority in the 80S fraction. The distribution of template activity for another relatively well-resolved polypeptide, P-33 (see Figure 7), was also determined. About equal amounts of mRNA were present in the four fractions in this case (Table VI).

The distribution of mRNAs in the light particle fraction is interesting. This fraction appears to contain a more limited population of abundant species, all apparently with a poly(A) segment [compare lanes IV of total and poly(A)$^+$ RNAs in Figure 7]. There are insignificant amounts of actin mRNA in this fraction.

Discussion

The size distribution of poly(A) segments in the actin mRNA of mouse sarcoma-180 ascites cells appears to be quite different from that of the other abundant mRNA species in these cells. A considerable portion of this RNA fails to bind to oligo(dT)-cellulose, and actin is the only major polypeptide synthesized in a wheat germ cell-free system primed by the unadsorbed polysomal RNA fraction.[2] The actin mRNA not bound to oligo(dT)-cellulose is designated in this report as poly(A)-deficient or poly(A)$^-$ RNA, although short homopolymer segments might be present in this material. Assays for poly(A) in the unadsorbed fraction by annealing with radioactive poly(U) (Jeffery & Brawerman, 1974) were negative. This test, however, involves trichloroacetic acid precipitation of the RNase-resistant poly(U) fragments, and can detect only poly(A) sequences large enough to protect acid-insoluble poly(U) chains. The minimum size of poly(A) segments capable of binding to oligo(dT)-cellulose has been estimated as about 30 nucleotides (Nudel et al., 1976) or 15–20 nucleotides (Levenson & Marcu, 1976). It can vary with the conditions of binding (Nudel et al., 1976). In the present study, the ionic composition of the binding buffer (0.5 M NaCl and 5 mM $MgCl_2$) was designed to enhance duplex stability. Thus, the poly(A) sequences in the unadsorbed RNA might be expected to be relatively short.

The size distribution of poly(A) segments in the oligo(dT)-cellulose bound actin mRNA also appears to be atypical. A large portion of this material fails to bind to Millipore filters. Only relatively long poly(A) segments (with a minimum size of 50–60 nucleotides) can bind to this adsorbent (Gorsky et al., 1974). The relative actin mRNA content of the Millipore-bound fraction is much lower than that of other poly(A)-containing species. Thus, the poly(A)$^+$ actin mRNA appears

[2] The conditions of gel electrophoresis used in this study would exclude the histones, which should also be coded for by the unadsorbed RNA fraction.

to have a relatively high proportion of chains with poly(A) segments in the 15-60 nucleotide size range.

The occurrence of the poly(A)-deficient actin mRNA raises some interesting questions concerning the control of poly(A) metabolism. This RNA component could represent the transcription product of a separate gene, lacking a signal that calls for poly(A) addition. It is also possible that the two actin mRNA components represent the same structural gene. In this case, the transcript might be polyadenylylated in normal fashion but be more susceptible to the cytoplasmic processes that lead to size reduction and loss of the poly(A) segment. The latter possibility seems more likely, since it could account for the apparent distribution of poly(A) sizes observed in the actin mRNA. It is known that two species of actin, separable by isoelectric focusing, occur in the cytoplasm of various mammalian cells other than muscle cells (Whalen et al., 1976). In the present study, the translation products were not analyzed by isoelectric focusing. Several investigators, however, have shown that the same actin species, β-actin, is synthesized in cell-free systems under the direction of either the poly(A)$^+$ or the poly(A)$^-$ mRNA (Hunter & Garrels, 1977; Kaufmann et al., 1977). It is shown here that the two actin mRNA components of Sarcoma-180 cells have a common structural feature that causes them to be highly susceptible to inhibition of translation in the presence of excess poly(A)$^+$ mRNA. It is likely that they too represent the same structural gene. The unusual response of the actin mRNA to the cellular processes that control poly(A) size would tend to indicate that these processes have the potential of being selective toward different mRNA species.

The actin mRNA also shows a distinct functional characteristic. Its translation is strongly inhibited in the presence of saturating amounts of poly(A)$^+$ RNA. This behavior is not related to the unusual poly(A) size distribution of the actin mRNA, since both the poly(A)$^+$ and poly(A)$^-$ components are susceptible to the inhibitory effect. The use of excessive amounts of mRNA in the cell-free system leads to a situation where mRNA molecules must compete for limiting amounts of ribosomal initiation process (Sonenshein & Brawerman, 1976, 1977). The actin mRNA would appear to be a relatively inefficient species in this respect. This interpretation, however, may not be quite correct, since homopolymers used as inhibitors of initiation failed to affect preferentially actin synthesis. It has been shown that inhibitory levels of synthetic poly(A) can have a differential effect on the translation of efficient and nonefficient mRNAs (Lodish & Nathan, 1972; Sonenshein & Brawerman, 1976, 1977).

The present study was undertaken with the assumption that the poly(A)-deficient actin mRNA that fails to bind to oligo(dT)-cellulose may represent a distinct functional entity. It remains to be determined whether RNA chains that may have oligo(A) sequences of 15-20 nucleotides can still be considered as poly(A)-containing mRNA from the functional point of view. It is known that the poly(A) segment in cytoplasmic mRNA is associated with protein (Kwan & Brawerman, 1972). Presumably, the protein component is essential for the function of this segment, whatever this function might be. One may wonder whether the protein(s) could remain associated with the poly(A) when the latter becomes very short. Experiments with globin mRNA injected into frog ooctyes strongly suggest that the poly(A) sequence serves to protect the functioning mRNA from degradation (Huez et al., 1974). Globin RNA chains with less than 30 adenylate residues at the 3′ end are not protected in this system and behave like RNA chains completely depleted of poly(A) (Nudel et al., 1976). Thus, the oligo(dT)-cellulose fractionation may be effective in resolving functionally distinct mRNA components.

The persistence in the cytoplasm of actin mRNA chains without poly(A) segments may be a fortuitous occurrence without functional significance. On the other hand, it may be the basis for a distinct physiological process affecting actin synthesis. The poly(A)-deficient actin mRNA appears to be functional, since it cosediments with polysomes in the mouse sarcoma cells as well as in other mammalian cells (Hunter & Garrels, 1977). Its apparent concentration in polysomes smaller than those containing the poly(A)$^+$ actin mRNA (see Table VI) may perhaps indicate that it has a lower initiation efficiency. The poly(A)$^-$ protamine mRNA component of trout testes also appears to be functional in vivo (Iatrou and Dixon, 1977). It is hoped that studies of the physiological control of actin synthesis, currently in progress in our laboratory, may reveal features that could be related to the poly(A)-deficient mRNA component.

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Size Distribution of Polyadenylated Adenovirus 2 RNA Synthesized in Isolated Nuclei†

Stephen G. Zimmer,‡ Carlos J. Goldenberg, David P. Carlson, Elizabeth A. Craig,§ and Heschel J. Raskas*

ABSTRACT: Viral RNA synthesis was studied in nuclei isolated 14 h after adenovirus 2 infection of KB cells. RNA synthesized in vitro was approximately 40% viral and was derived almost exclusively from the *r* strand (95%). Approximately 30% of the nucleotides incorporated in vitro bound to oligo(dT)-cellulose. These values are comparable to those obtained for RNA synthesized in vivo. Hybridization-inhibition experiments demonstrated that the in vitro transcripts include two classes of sequences, those restricted to the nucleus in vivo and those present in cytoplasmic RNA. Quantitation of viral sequences from defined regions of the genome was accomplished by exhaustive hybridization. Hybridization was performed with seven viral DNA fragments generated by digestion with either endo R·*Eco*R1 or endo R·*Sma*I. Of the regions tested, the most abundant RNAs were derived from genome positions 39–52 and 77–91. Some regions of the genome were transcribed in eightfold greater amounts per unit length than other regions. Similar results were obtained with RNAs labeled in vitro and in vivo. The size distribution of polyadenylated viral RNA in nuclei incubated in vitro was examined by two types of experiments. First, the sizes of viral RNAs synthesized in vitro were determined, and in other experiments size distributions were determined for viral RNAs labeled in vivo and then incubated in vitro. Polyadenylated viral RNAs synthesized in vitro ranged in size from 13S to 28S with peaks of 28S, 25S, and 22S RNAs superimposed on a heterogeneous background. The polyadenylated nuclear RNA labeled during a 30-min in vivo pulse was considerably larger than the in vitro product, varying in size from 23S to 36S. Incubation of the prelabeled nuclei in RNA synthesis conditions resulted in a gradual size change of the prelabeled RNAs to a distribution similar to that of the in vitro products. *Eco*R1 fragments E (83–89) and C (89–100) were used to perform a more detailed analysis of nuclear RNAs transcribed from regions encoding the 22S fiber mRNA. Hybridization with both fragments revealed that discrete 28S, 25S, and 22S RNAs were synthesized in vitro. RNA prelabeled in vivo contained 36S, 28S, and 22S RNAs which were converted during in vitro incubation to a size distribution identical to that of the in vitro products. Such in vitro systems offer the possibility of studying coupled mRNA transcription and processing.

Studies of viral RNA synthesis in human cells productively infected with adenovirus 2 have elucidated differences between the nuclear transcripts and the cytoplasmic mRNAs. The cytoplasmic RNAs synthesized late in infection consist of a set of at least 12 mRNAs which are polyadenylated at the 3′ end and capped at the 5′ terminus (McGrogan and Raskas, 1977; Chow et al., 1977a; Gelinas and Roberts, 1977). In contrast, the nuclear fraction consists of a heterogeneous population of poly A(+) and poly A(−) molecules (Philipson et al., 1971). The nuclear RNAs include molecules significantly larger than the largest cytoplasmic mRNAs (Parsons et al., 1971; Bachenheimer and Darnell, 1975); some molecules may be transcripts of a DNA segment as large as 80% of the 35 000 nucleotide viral genome (Meissner et al., 1977; Weber et al., 1977). In addition, the nuclear RNAs contain sequences that are not present in the cytoplasmic fraction (Thomas and Green, 1969; Philipson et al., 1974; Sharp et al., 1974).

At late times in infection, after the onset of viral DNA synthesis, greater than 95% of the newly synthesized viral mRNA is derived from the viral *r* strand (Pettersson and Philipson, 1974). Several types of experiments have provided evidence that the large nuclear transcripts are precursors of these mRNAs and that a major promotor for rightward transcription is located at approximately map position 20 on the viral genome (Bachenheimer and Darnell, 1975; Weber et al., 1977; Goldberg et al., 1977; Evans et al., 1977). Recent structural studies of the late viral mRNAs have demonstrated the existence of a 150–200 nucleotide sequence common to the late mRNAs transcribed from map positions 20–100 (Chow et al., 1977b; Berget et al., 1977; Klessig, 1977). This leader sequence is located at the 5′ end of the mRNAs and includes sequences from genome positions 17, 20, and 27. This finding provides further evidence that the region around position 20 constitutes the major promotor for late rightward transcription.

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‡ Current Address: Department of Pathology, University of Kentucky Medical Center, MDRF 3, Lexington, Ky. 40506

§ Current Address: Department of Biochemistry and Biophysics, University of California School of Medicine, San Francisco, Calif. 94144

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